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14. ABSTRACT

This report presents a summary of the research activities, major accomplishments, presentations supported by ONR grantN00014-09-C-0583 to Advanced Brain Monitoring, Inc. Key contribution of this project was to assess the efficacy of Omega-3 fatty acids (EPA/DHA) in mitigating the cognitive,mood, and performance aspects of sleep loss. Both acute sleep deprivation of 48 hr in a civilian populaiton and ongoing sleep chronic sleep debt accumulation in a military popuation were investigated. Results found that Omega-3 fatty acids were able to preserve baseline performane, mood and neurophysiology through 36-39 of complete deprivation. In the military populaiton, undergoing naturally occuring sleep loss, Omega was also able to support improved performance compared to placebo, up through an average of 31 hr of sleep loss. Additional physiological metrics from EEG and cytokines support the hypothesis that the improved perfroamnce is due to reduced inflammatory activity with Omega supplementation. These data suggest that Omega-3 fatty acids provide a viable option to reduce the effects of sleep deprivation that naturally occurs throughout military training and operations, however additional data regarding performance in military relevant tasks would be required before implementation force-wide.

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Summary

This report presents a summary of the research activities, major accomplishments, presentations supported by ONR grantN00014-09-C-0583 to Advanced Brain Monitoring, Inc. Key contribution of this project was to assess the efficacy of Omega-3 fatty acids (EPA/DHA) in mitigating the cognitive,mood, and performance aspects of sleep loss. Both acute sleep deprivation of 48 hr in a civilian populaiton and ongoing sleep chronic sleep debt accumulation in a military popuation were investigated. Results found that Omega-3 fatty acids were able to preserve baseline performane, mood and neurophysiology through 36-39 of complete deprivation. In the military populaiton, undergoing naturally occuring sleep loss, Omega was also able to support improved performance compared to placebo, up through an average of 31 hr of sleep loss. Additional physiological metrics from EEG and cytokines support the hypothesis that the improved perfroamnce is due to reduced inflammatory activity with Omega supplementation. These data suggest that Omega-3 fatty acids provide a viable option to reduce the effects of sleep deprivation that naturally occurs throughout military training and operations, however additional data regarding performance in military relevant tasks would be required before implementation force-wide.

I. Introduction

Substantial research has been done on the effects of sleep deprivation, in academia, the private sector, and by multiple defense organizations. This extensive research has shown us that sleep deprivation leads to many adverse outcomes observable in both the laboratory and in field environments. Sleep deprivation has both cognitive/behavioral and physiological consequences. The behavioral consequences of sleep loss include: slowed reaction time and increased errors (1-5), disruption in declarative and procedural learning (6). Sleep deprivation is associated with physiological imbalances as well, such as elevated pro-inflammatory cytokines (7-13). For our military troops, the consequences of these effects have a great potential to reduce the efficacy and efficiency of our forces. Having reduced reaction time and impaired accuracy of situational awareness increases the likelihood of injury in combat (and non-combat situations as well). Once injured, the aberrant inflammatory milieu can lead to inappropriate immune responses that may increase likelihood of opportunistic infections and will reduce wound healing (14-15). During training, reduction in learning and memory function reduced the rate of training and long term retention. In addition, long term physical and mental health concerns may also arise, given that pro-inflammatory cytokines are related to accelerated aging of the immune system, acceleration of age related degeneration of tissue, and depression (16-18).

Advanced Brain Monitoring has been a contributor to the field of sleep medicine for over ten years, developing EEG-based cognitive assessment tools to evaluate the effects of both chronic and acute sleep loss(19-20). Recent data from Advanced Brain Monitoring (ABM) provide further evidence for the adverse consequences of chronic sleep deprivation. Accumulation of sleep debt also negatively impacted basic performance metrics during USMC Mojave Viper training exercise (19). ABM assessed the effects of sleep debt in a group of Marines participating in the Mohave Viper training program at the Twenty-nine Palms desert warfare training facility. USMC battalion/platoon leaders (n=17) were evaluated during the 21-day, live-fire training exercises with daily continuous actigraphy and weekly wireless EEG and heart rate / heart rate variability (HR/HRV) acquired during a 20-minute, 3-Choice-Vigilance-Test (3C-VT). In the 3C-VT, subjects are required to identify “target” vs. two “non-target” stimuli that are presented at varying intervals over the 20-minute session. These data demonstrated that Marines become impaired due to sleep debt, but are unlikely to acknowledge any impairment subjectively.

Interestingly, pro-inflammatory cytokines alone are associated with cognitive and behavioral effects similar to sleep loss. Slowed reaction time has been associated with elevated pro-inflammatory cytokine activity in multiple human (21-22) and animal studies (23-26). Cognitive accuracy has also been associated with elevated cytokine levels (23-24, 27-29). Procedural and declarative learning are also thought to be impaired by pro-inflammatory activation (21, 24, 30-34). While a great deal of work on cognitive impairments associated with cytokine activity began in animal models (16), this work has been replicated in human work as well(35-41). Based on these associations: sleep loss is associated with elevated pro-inflammatory cytokine activity(7, 42-44), and elevated pro-inflammatory cytokine activity is associated with many of the cognitive deficits associated with sleep loss(45-65), it would be reasonable to hypothesize that the cognitive/behavioral effects of sleep loss may actually be caused by the increase inflammatory stimuli associated with sleep loss. Therefore, the hypothesis of this proposal is that the physiological effects of sleep deprivation may in fact be the underlying cause of the cognitive/

behavioral effects, and by addressing the physiological issues, we may be able to also address the cognitive/behavioral issues.

The most effective strategy for sleep loss amelioration (including decreasing pro-inflammatory cytokine levels) has been shown to be napping (6, 13, 60, 66-67), however access to naps in the field is inconsistent and unpredictable. Therefore, alternative mitigation strategies must be investigated. Intervention strategies must take into account the deployment context: interventions that inhibit the efficacy of the soldier are not viable. Several pharmaceutical options are available to ameliorate the effects of sleep loss, however the side effects are generally unacceptable for use in the field (these included significant sleep inertia, nausea, diarrhea, anxiety, dizziness). There are also recent discoveries for direct pro-inflammatory suppression using drugs such as ketamine(68-69) and Haloperidol(70), however these too have significant and unacceptable side effects for field deployment, including vision problems, confusion, drowsiness, and loss of control of limb movement. It is quite common for soldiers and marines to self-medicate through sleep loss with stimulants such as caffeine, amphetamines, methylphenidate, and possibly even narcotics. However these strategies have significant contra-indications. Narcotics have well known cognitive and behavioral side effects that are detrimental to the overall mission goals of the military, as well as being illegal, so these are not an option. Amphetamines, methylphenidate (Ritalyn) and Modafinil (Provigil) are legal pharmaceutical stimulants. Amphetamines and methylphenidate are both addictive, and while it may mask the feeling of fatigue, the behavioral and cognitive effects of sleep loss are still likely to manifest (71). Modafinil is an option for mitigating the effects of sleep loss, as the side effects are not as severe; however, in doses high enough to be adequate to mitigate the effects of significant chronic or acute sleep loss, it is often associated with nausea and headaches, reducing the effectiveness of the soldiers. Caffeine is already in use, and in fact abused not only by the military, but by the general population, however caffeine in high doses has many adverse side effects as well. First, the body adapts to caffeine regularly, requiring ever increasing amounts in order to be effective. Second, at high doses, it can alter perception, increase shakiness, and detrimentally effect regular sleep patterns. Finally, caffeine in high doses can have long term negative effects on bone density, and muscle tone, as well as increasing inflammatory stimuli in the body- leading to the negative effects of long-term exposure to inflammation discussed earlier. These include advanced aging of the physical and immune systems, and increased risk of depression. With this in mind, better alternatives may be nutriceuticals: foods with added, often drug-like, benefits to health and well being.

There are many nutriceuticals that may be effective in reducing the effects of sleep loss through reduction of the effects of pro-inflammatory cytokines, and synergize the effects of napping, including: vitamin E, vitamin C, Turmeric, Resveratol (found in red wine), Green Tea, Black Tea, Genisten (found in soybeans), Chamomile, Ginseng, feverfew, and probiotic yogurt(72-74). As experimental interventions for reducing the effects of sleep loss, several of these are inappropriate: both green and black tea contain caffeine that may interfere with sleep when available, vitamin intake is difficult to monitor and account for outside the experimental condition (although given the simplicity of this intervention, it should be examined in the future as well), and several of the other options have not been shown to be effective in vivo. In contrast, Omega-3 fatty acids (ω3FAA) have been shown to effectively reduce inflammatory states, acting on the mechanisms that result in cytokine production in ex vivo and in vivo

models, as well as in both animal and human (74-83). Sleep quality may also benefit from ω3FAA, although this is not definitive(84). Therefore, the mediating interventions will include both Omega-3 fatty acid (ω3FAA) treatment in isolation and paired with napping, in order to evaluate the synergynism that the ω3FAAs may provide to the napping.

In order to address the growing military need to mitigate the effects of sleep loss in order to maintain a fit and ready force, as well as improve quality of life in both the short and long term for our troops, the following parallel studies were conducted to address three goals: 1) assess the effectiveness of Omega-3 fatty acids in mitigating the effects of acute and chronic sleep deprivation, 2) assess inflammatory activity throughout both acute sleep deprivation in order to begin to determine if the effects of sleep loss are primarily mediated through inflammation, 3) compare how acute and chronic sleep loss compare on identical metrics to develop "standardized curves" for use by military leaders in evaluating the majority of studies that are conducted using the acute sleep loss model, and how it might apply to military populations that undergo chronic sleep loss.

These studies were conducted with civilian volunteer for the acute study, while the chronic study was conducted with the Recon Training Unit at School of Infantry-West, at Camp Pendleton under COs Byrne and Wooley.

II. Methods

2.1. Participants

2.1.1. Acute. A sample of n=38 participants were enrolled after screening for the following exclusion criteria: 18-28 yr, physically fit (BMI ≥25), and physically active (3X or more per week of cardiovascular exercise for at least 30 min), self report of excessive daytime sleepiness (Epworth > 6), excessive smoking (more than 10 cigarettes/day) caffeine intake (more than 5 cups/day) or alcohol use (5 r more drinks per day), history of sleep, neurological or psychiatric disorder, head trauma, symptoms of a sleep disorder, and inconsistent sleep patterns (> 7.25 hr/night on average). The final complete data set consists of the N=30 that completed both sleep deprivation sessions. Table 1 provides the demographic data for this population.

Table 1. Acute study demographics

	Male	Female	Total
# of participants	20	10	30
Age	22.26 (Range 18-26)	23 (Range 19-25)	22.26 (Range 18-25)
BMI	22.1 (range 19-25)	21.75 (Range 19-24)	22.1 (Range 19-24)

2.1.2. Chronic. A sample of 67 Marines enrolled in the Recon Training Course at SOI-W on Camp Pendleton was recruited to participate in this study. Subjects were recruited during MARC (Marines awaiting Recon Training), prior to the course beginning. The same exclusion criteria were applied, except for age limit, physical activity requirement and BMI, as these are assumed in the population. The final dataset is limited to an n=20 as this is the population that completed the class and the protocol,

lasting 12 weeks. However, data analysis was completed on both subjects that dropped, up until they dropped, and those that completed the entire class and study. Partial completion data was acquired on n=50 at baseline, n=35 at session 1 (5-6 weeks into the course), n=28 at session 2, n=23 at session 3, n=22 at session 4, n=21 at session 5. These data are included in the univariate ANOVAs that were used as follow on analysis, and are included in the graphs, and acute vs. chronic analysis. However, only complete data were included in the repeated ANOVAs.

2.2. Materials/Equipment

2.2.1. Omega-3 fatty acids/Placebo. Omega-3 fatty acids were acquired from NutraOrigin, Inc. Each active 1000 mg pill contains: 300 mg of EPA, 200mg of DHA, 100 mg of other Omega-3 fatty acids, and the other 400 mg of ingredients are: gelatin, glycerin, and mixed tocopherols. Participants took 2 gel tablets with both the morning and evening meal, for a total dose of 4000mg, with 1200 mg EPA/800 mg DHA. The placeboes were acquired from Seagate, Inc. The placebo gel tablet was also 1000 mg, and identical in appearance to the active tablets. Each placebo gel tablet contained: 800 mg of monounsaturated fat, which is almost entirely composed of Oleic Acid; plus 5 mg natural Vitamin E added for antioxidant stability; 5 mg of Omega-3 (Linoleic Acid); and 190 mg of saturated and polyunsaturated fats that are naturally-occurring in olive oil. Participants on the placebo also took 2 gel tablets with both the morning and evening meal, for a total dose of 4000 mg.

Olive oil is one of several placeboes used in Omega-3 fatty acid literature. This placebo was chosen for this study because of the hypothesis that inflammation may be related to the effects of sleep loss. Olive oil is more inflammatory neutral than other placeboes used which have included corn oil and mixed vegetable oils, both of which are high in Omega-6 fatty acids, potentially mediating the inflammatory response.

2.2.2. Subjective measures. Subjective assessments of sleep, mood, and wellbeing were acquired throughout the study. These measures included: the Beck Depression Index, the Center for Epidemiology Studies- Depression Scale, the Profile Of Mood states, the Medical Outcome Survey- 36 item, the State-Trait Anxiety Index, the Pittsburg Sleep Quality Index (respectively: BDI, CESD, POMS, MOS-36, STAI, and PSQI).

The BDI is a 21 item multiple choice self report inventory with substantial validation studies completed over the past 40+ years, while the most current version has been in use for over 10 years. The metric is used by both clinicians and researchers, to assess risk of depression. Each question is scored from 0-3, and total scores relate as follows: 0–13: minimal depression; 14–19: mild depression; 20–28: moderate depression; and 29–63: severe depression. Higher total scores indicate more severe depressive symptoms. *Note: The BDI was not used for the chronic study due to the inclusion of suicidal ideation items that would require reporting up the chain of command and reduce subject privacy.*

The CESD is a depression metric developed at NIH in 1977, that includes 20 items (but does not include suicidal ideation items that are included on the BDI). This scale also has a rich history of validation studies; however it is primarily used in research studies, to provide a range of depressed mood- it is not intended as a screening for major depression. As with the BDI, each item is scored from 0-3, with total score being indicative of increased risk, the range being 0-60. The ranges of scores are: 0-

16: average; 16-24: borderline elevation of depressive symptoms; 24 and above: significant elevation of depressive symptoms.

The POMS is a 65 item general mood scale often used in research with well validated scales for five factors: Tension/Anxiety, Depression, Anger/Hostility, Vigor, Fatigue, and total that is indicative of overall mood (calculated by summing: Tension + Depression + Anger + Fatigue - Vigor). Each item is scored from 0-3. Higher scores on each scale indicate a more negative mood state, with the exception of vigor.

The STAI is a 40 item scale to assess anxiety that was developed in 1970, published in 1983, and has been used primarily in research studies. The primary purpose of the STAI is to distinguish anxiety from depression. Higher scores indicate higher anxiety, however, normal ranges are not provided by the authors (scores range from 0-80).

The MOS-36 is a 36 item index with measures of overall well being, health status and change in health status. There are 9 scales that result that include: Physical functioning, Role functioning- physical, Role functioning- emotional, energy/fatigue, emotional well being, social functioning, pain, general health, and health change.

The PSQI is a sleep quality assessment that includes 19 self-scored items that are included in the scoring. This metric was developed in 1989. Multiple validation studies have been completed, and this metric is primarily used in sleep research and research oriented sleep laboratories. Each item is scored 0-3 and the range of scores considered normal is anything under 5.

2.2.3. Actigraphy. Each participant wore an actigraph (#OBMA-0.2, PCDI, Florida; ReadiBand, Fatigue Science, Hawaii) for one week prior to each experimental session to track sleep/wake activity levels, and to confirm compliance with the sleep deprivation requirements in the acute study, and to track amount of sleep debt for the chronic study.

2.2.4. B-Alert EEG Sensor Headset/ AMP Neurocognitive test bed. The Sensor Headset was developed by Advanced Brain Monitoring as a portable system to record both electroencephalographic (EEG) and electrocardiographic (EKG) signals. The headset, made from stretchable fabric, fits snugly on the head and houses EEG sensors. The EKG leads are attached to the upper right clavicle and lower left rib. The main electrical component of the Sensor Headset is a battery-powered data acquisition and transmission unit that rests against the back of the head. The Sensor Headset is only used for recording physiological signals and does not introduce energy into the body except for any minor electromagnetic radiation typically emitted by small electronic devices. The only risk posed by this device is mild discomfort due to the pressure exerted by the cap and sensors on the user's head. To minimize this risk, caps are available in three sizes and are adjustable.

Figure 1. The B-Alert Sensor headset.

The sensor headset is integrated with the neurocognitive test battery to allow simultaneous acquisition of EEG (8 channels Fz, Cz, POz, F3, C3, C4, P3, P4), performance, and heart-rate data during tests of vigilance, attention and memory in the Alertness and Memory Profiling System (AMP). The AMP uses a multivariate approach that allows simultaneous acquisition and analysis of data that requires days or even weeks with conventional laboratory methods. The brief AMP consists of four tasks: the 3 Choice Vigilance Task (3C-VT), Eyes Open vigilance task (EO), Eyes

Closed vigilance task (EC), and Standard Image Recognition (SIR) tests. We are also evaluating adding the multiple object tracking task, as a working memory task.

2.2.5. AMP Task details. The AMP test battery consists of four tasks:

1. The **3-Choice Vigilance Task (3C-VT)** incorporates features of the most common measures of sustained attention, such as the Continuous Performance Test, Wilkinson Reaction Time, and the PVT-192, and it was designed to allow simultaneous monitoring and quantification of the EEG. The 3C-VT requires subjects to discriminate one primary (70%) from two secondary (30%) geometric shapes presented for 0.2 seconds over a 20-minute test period. A training period is provided prior to the start to minimize practice effects. The 3C-VT challenges the ability to sustain attention by increasing the inter-stimulus interval (ISI) across four, 5-minute quartiles. During the first 5-minute quartile, the ISI ranges from 1.5 to 3 seconds, increasing up to 6 seconds during the second 5-minute quartile, and up to 10 seconds during the third and fourth 5-minute quartiles, for a total of 20 minutes for the entire task. Subject's performance during the 3C-VT has proven sensitive to the effects of full and partial sleep deprivation in healthy subjects and to excessive daytime sleepiness in patients with Obstructive Sleep Apnea(85-86). Concurrent validity was established in sleep deprivation studies by correlation with behavioral evidence as measured by: cessation of finger tapping; visually scored observations of facial signs of drowsiness (eye closures, head nods); responses to a subjective sleepiness questionnaire; visually scored EEG; modified MWT; handheld PVT-192 test; and driving simulator performance (85).

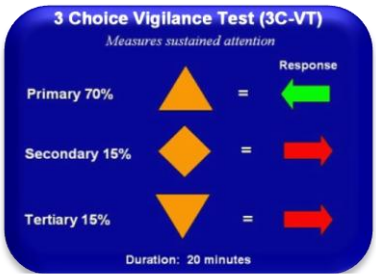

Figure 2. Example of an instruction screen for the 3CVT.

2. Each AMP session includes a **5-minute Eyes Open (EO) and Eyes Closed (EC)** with paced button press. EO presents a 10 cm circular image for 200 milliseconds in the center of the computer monitor, repeating every two seconds. Participants are instructed to press the space bar each time they see the image. For EC, an auditory tone every 2 seconds prompts participant to press the space bar.

3. The **Image Recognition and Memory Test (IR) evaluates** attention, distractibility, encoding and image recognition memory. For the Standard IR, during the training session, a group of 20 images are presented twice. The testing session presents the 20 training images randomly interspersed with 80 additional images. Subjects must indicate whether or not the image was in the training set. Five equivalent image categories are available including animals, food, household goods, sports, and travel.

AMP Metrics: The AMP results in performance, EEG, and EKG metrics. The performance metrics are percentage correct, incorrect, and missed (also referred to as lapses), as well as reaction time. EEG metrics are classic bandwidths (delta, theta, alpha, beta, gamma and sigma) and our proprietary algorithms from drowsiness to alertness. These metrics were developed by having a large population complete tasks that were accepted in the literature as requiring a) almost no attention (EC), b) a small amount of attention (EO), and c) a great deal of attention (3CVT). Then certified experts in sleep medicine scored each of these EEG sessions and identified epochs they considered to be indicative of sleep. Those epochs that had consensus were used to anchor the model's low end as sleep onset, while those epochs that were not agreed upon were removed from the development analysis. A four class quadratic discriminate function model was then trained to identify the four classes: sleep onset,

distraction, low engagement, and high engagement. The training data included n=160 healthy subjects, with at least ten subjects in each decade of age from 18-70(85).

EKG metrics are heart rate and heart variability measures that include low frequency variability, high frequency variability and the ration between low and high frequency. Low frequency is associated with sympathetic activation and anxiety (87-92), while HRV in general has correlations with cognitive function that are only recently being discovered and elucidated (91).

2.2.6. Blood Spots. Blood Spots were collected using finger pricks at Baseline, and at the end of each session (for acute) or class (for chronic). A 21g UniStick (Murietta, GA) was used to prick the middle finger of the non-dominate hand, and 7 full spots were collected according to standard procedures on Whatman 903 filter paper card (with 5 spots on each card, Piscataway, NJ). Blood was then allowed to dry at room temperature, and stored with a desiccant packet until shipment for assay. Five spots were used for Multiplex assay for a panel of inflammatory cytokines (Rules Based Medicine, Austin TX), and 2 spots from a second card were submitted to OmegaQuant (Sioux Falls, SD) for assessment of the Omega-3 level across conditions.

2.2.7. Saliva. Saliva was collected at the same time points as blood spots and submitted to ELISA for a panel of cytokines by the Shaw laboratory at Washington University, St Louis. Salivary collection consisted of asking participants to chew on the cotton swab part of the Salivette (Sarstedt 51.1534, Newton NC)

2.3. Protocols

2.3.1. Acute. The acute study consisted of a double-blind, cross over design. Thus each subject had three sessions. The initial session includes the orientation to the study, informed consent, and then collection of fully rested assessment of all metrics, including the AMP/EEG, subjective metrics, and assignment of sleep and food logs, and pills. The next two experimental sessions were identical, with the exception of the placebo or Omega-3 treatment. The condition/pill assignment for the first session was randomly assigned, with half of the subjects beginning with Omega-3 fatty acids, and the other half placebos. The condition/pill assignment was switched for the second session. The timing of the three sessions is as follows: orientation, take pills for 4-6 weeks, session 1, take second set of pills for 4-6 weeks, session 2. Each experimental session included the last 24 hours of a 48 hour period of sleep deprivation. One week before each experiment session participants were sent an actigraph to wear for the week to determine regular sleep cycles, and to confirm compliance with the sleep deprivation requirements. In addition, participants were required to call a voice mail line every 2 hours during regular working hours and every 30 minutes thereafter for the 24 hours of sleep deprivation that occurred prior to arrival at the office.

Once subjects arrive, the participants filled out the subjective measures and began the first cycle of AMP (3CVT, EO, EC, and SIR). In addition to these data presented herein, subjects also completed the Automated neuropsychological Assessment Metrics (ANAM) administered upon arrival (24 hr sleep deprived), at the diurnal dip (30 hr sleep deprived), and during the last cycle (48 hr sleep deprived). This schedule allowed for 8 identical cycles (with unique stimuli to reduce learning effects) of AMP to occur approximately every 3 hours, with a minimum of 60 minute breaks in between each cycle. After cycle 5, participants were also allowed to take one 40 minute “powernap”.

2.3.2. Chronic.

The chronic protocol consisted of a between subjects, double blind design with repeated evaluations, including a fully rested baseline (during MART, Marines awaiting recon training, the week prior to the course beginning) and six repeated assessments throughout the last half of the 12 wk training. Recruitment occurred during MART, at 1600-1800, where all available potential class members were given a description of the study and asked to volunteer (only students were present to ensure freedom of choice for the Marines). Those willing to participate were asked to stay and underwent informed consent in a private area. Once informed consent was obtained, biomarker samples were collected to ensure that no diurnal differences would confound the data collected at the end of the course, which required evening collection. The following morning at 0800-1000 participants completed the AMP and self report baselines. At this point participants were assigned to either the BLUE or ORANGE groups (in order to maintain double blind nature of the design) and asked to take pills from the appropriately color coded bottles on a daily bases as described above, as well as fill out food and sleep logs daily, for the remaining 12 weeks of the course/study. Additional AMP and self report measures were then collected at 1700 every 7-10 days beginning midway through the training course and continuing until the end of the class. On the final data collection date, biomarkers were again collected. Only after final data analysis was completed did unblinding occur for presentation herein.

III. Results

3.1. Acute

3.1.1. Omega-3 Blood Lipid level. Omega-3 blood lips profiles were analyzed to ensure both compliance and to determine the efficacy of the intervention. ANOVA found that Omega-3 treatment lead to a significant elevation in blood lipid levels of Omega-3 fatty acids compared to placebo, $\underline{F}(1,27) = 4.02$, $\underline{p} < .01$. Figure 3 presents these data.

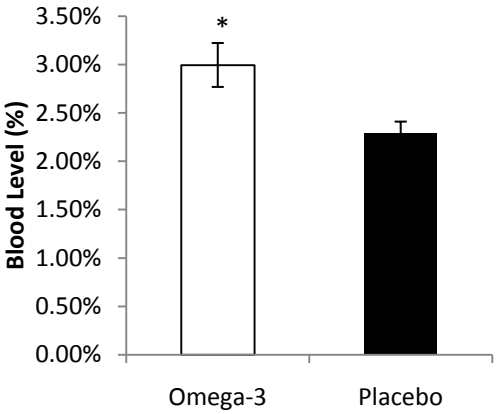

Figure 3. Omega-3 blood lipid % across treatment found that Omega-3 supplementation improved this metric.

3.1.2. Self Report Mood/Well Being. Self report metrics from the POMS, BDI, CESD, STAI, MOS-36, and PSQI were analyzed. Overall, there were few differences across groups or changes from baseline. Table 2 summarized these data, and includes published norms for comparison. Generally, this young, healthy population had better than normal/normal range of responses on all of these metrics, with the exception of the STAI. The STAI indicated that this population as a whole had slightly elevated anxiety compared to norms.

The only significant differences revealed by ANOVA were for POMS Vigor, $\underline{F}(2,86) = 5.96$, $\underline{p} < .01$ and Fatigue $\underline{F}(2,86) = 3.66$, $\underline{p} < .05$, although the overall trend in the means is for Omega treatment to lead to slight improvements. For Vigor, post hoc analysis revealed that placebo had significantly less

vigor compared to both Baseline and Omega. For Fatigue, Placebo treatment was associated with greater fatigue compare to both Baseline and Omega. These data are shown in Figure 4.

3.1.2. Performance. Lapses (failure to respond for 3 s or longer) during the passive auditory (EC) and visual (EO) vigilance tasks were examined. Repeated measures two-way ANOVA (treatment X time) found no significant differences ($p > .05$) over time, across treatment, or any interaction of time and treatment.

Accuracy and speed were analyzed for both the 3CVT and the IR tasks using Repeated ANOVA across the fully rested baselines and the eight sleep deprived time points. The repeated ANOVA (2 X 9) revealed that Omega-3 supplementation led to significantly better accuracy on both parameters for both tasks, Fs (8,219) > 2.79, $ps < .01$, but only marginally faster speeds Fs (8,219) > 1.68, $ps \leq .10$. These data are shown in Figure 5.

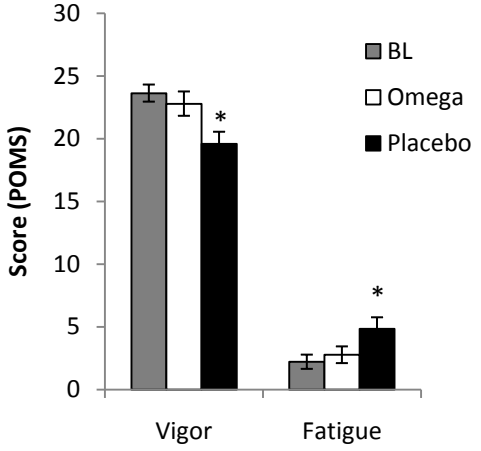

Figure 4. POMS Vigor and Fatigue scales across treatment conditions. Placebo treatment led to reduced Vigor and elevated Fatigue, however both were well within normal or better compared to published norms.

Table 2.

Measure	Scale	Norm	BL	Omega	Placebo	p
BDI	Depression	0-13				NS
CESD	Depression	0-16				NS
STAI	Anxiety	36 +/- 10				NS
PSQI	Sleep Quality	0-5				NS
MOS	Physical	>84.2				NS
	Role- Physical	>80.9				NS
	Bodily Pain	>75.2				NS
	General Health	>71.9				NS
	Vitality	>60.9				NS
	Social Functioning	>83.3				NS
	Role- Emotional	>81.3				NS
	Mental Health	>74.7				NS
POMS	Tension/Anxiety	<8				NS
	Depression	<8				NS
	Anger	<8				NS
	Vigor	>19	23.6	22.8	19.6	.05
	Fatigue	<8	2.2	2.7	4.8	.05
	Confusion	<6.5				NS

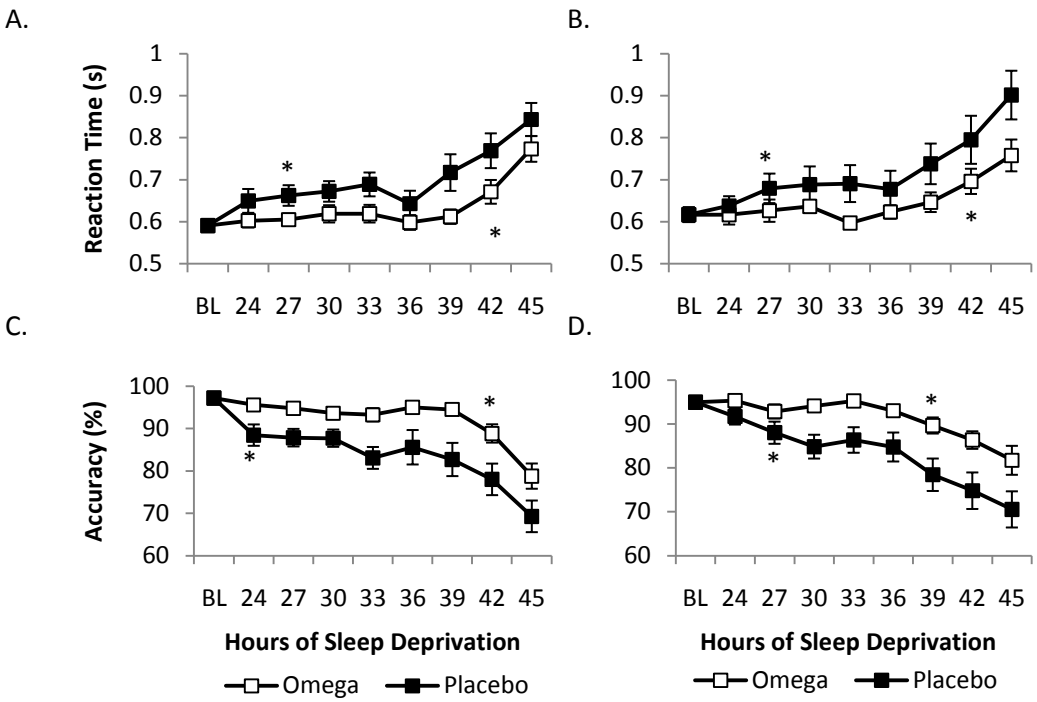

Figure 5. Performance metrics for 3CVT (A, B) and IR (C, D) including Speed (A, C) and Accuracy (B, D). Asterisks (*) Indicate the time at which performance decremented

Further analysis was conducted to determine when performance began to significantly decrement in comparison to baseline for both tasks. Individual ANOVAs (2 X 1) were conducted, and revealed that for the placebo condition, performance began to significantly decrement at 24-27 hr for both performance metrics on both tasks, Fs (1,27) > 6.43, ps < .05. However, this analysis conducted on the Omega-3 treatment group, found that baseline level performance was preserved for both metrics, across both tasks until 39-42 hr of sleep deprivation, Fs (1,27) > 6.52, ps < .05. These data points are indicated with an asterisk (*) in Figure 5.

Additional analysis was done across the 4 quartiles of the 3CVT in 4 separate repeated two way ANOVAS (treatment X time), 1 per quartile. A main effect of treatment, Fs(1,29) ≥ 18.15, ps ≤ .001; and time, Fs(8,219) ≥ 14.81, ps ≤ .01 occurred as early as quartile 2 for both speed and accuracy. However the interaction of treatment X time only occurred in the final quartile for speed, Fs(8,219) ≥ 2.21, ps ≤ .05. These data are shown in Figure 6.

Performance metrics for the ANAM tasks were also examined. A two way ANOVA (treatment X time) revealed no significant main effects for time or treatment, or any interaction of time X treatment. These data are shown in Figure 7.

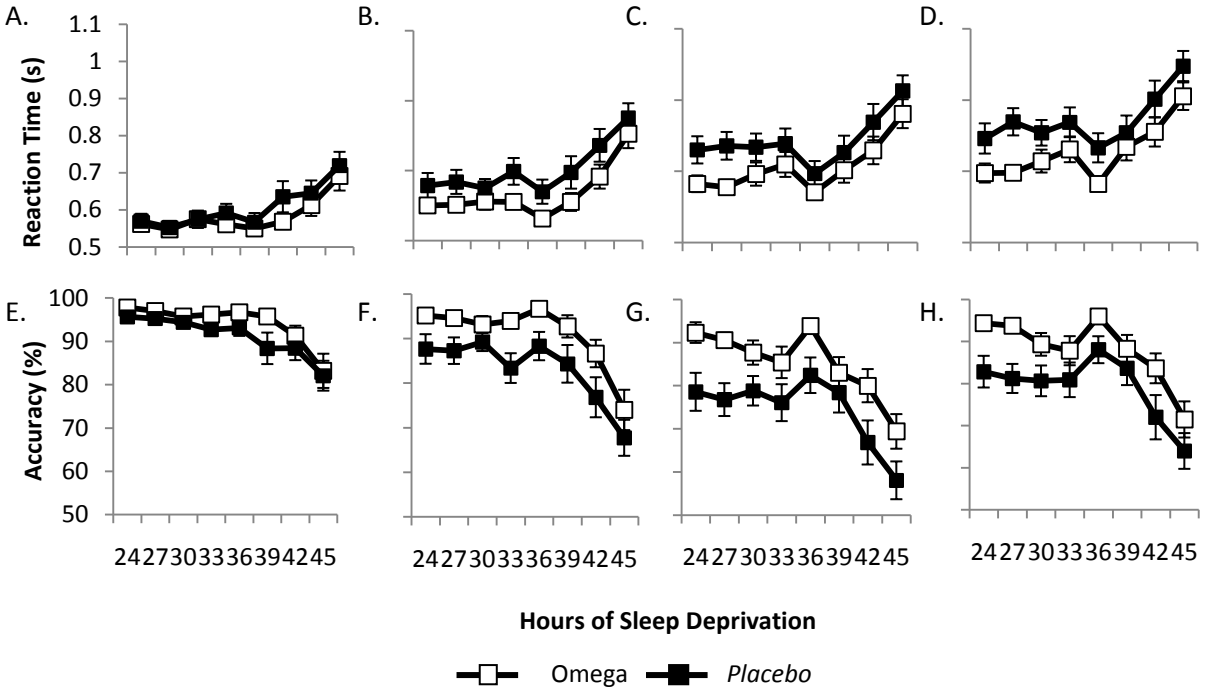

Figure 6. 3CVT performance metrics by quartile (20 min task examined in 5 minute sections). Reaction Time for Quartiles 1-4 are shown in A-D, while accuracy is shown in E-F. Main effects for time and treatment occur from Quartile 2 on (B, F), while interaction of time X Treatment occurs only in the last quartile (D, H).

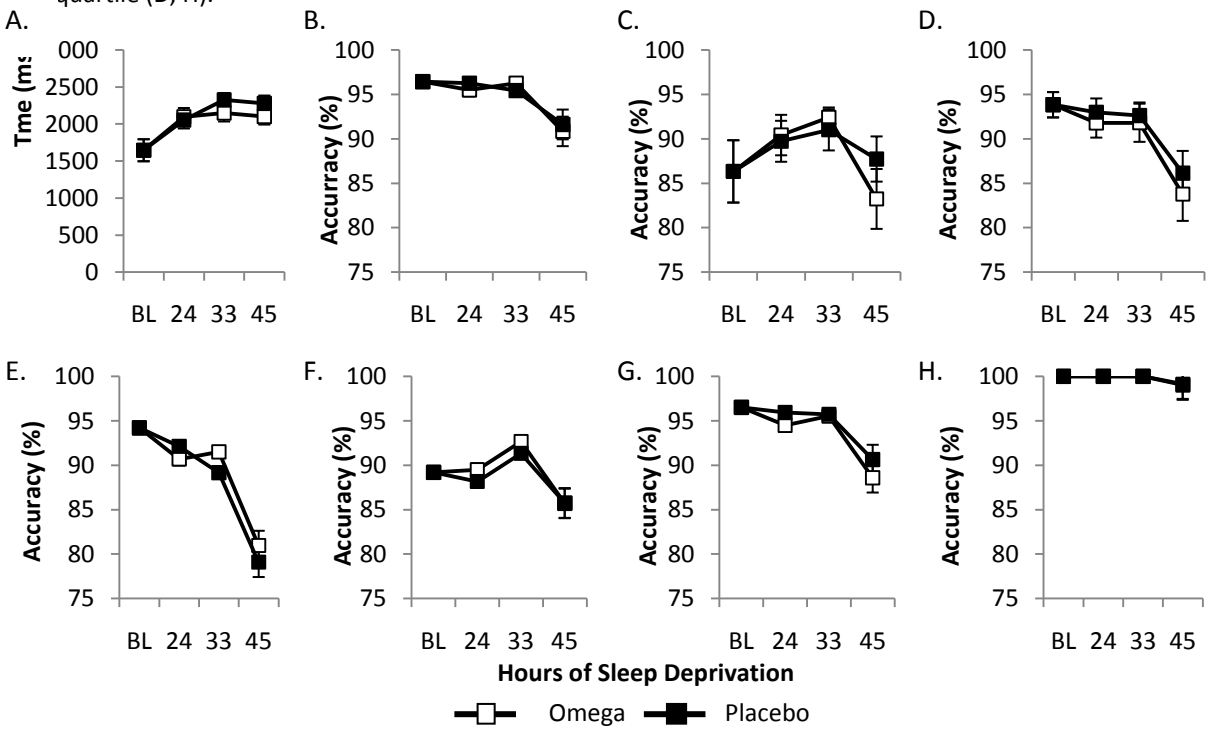

Figure 7. ANAM performance metrics for Tower puzzle (A), Code substitution (B), Delayed Code substitution (C), Match to sample (D), Logical reasoning (E), mathematical processing (F), Procedural response time (G) and Simple response time tasks (H).

3.1.3. EEG ERPs.

3CVT. For the 3CVT data, the ERP for the Correct Targets were calculated and the average amplitudes of the P300 feature (between 175-450 ms) were compared across treatments at baseline, and after 24 and 48 hr of sleep deprivation, for frontal, central, and parietal sites (Figure 8 A, B, C relatively). ANOVA found significant effects over time x treatment for frontal, F (2,78) = 4.24, p < .05, central, F (2,78) = 7.77, p < .01, and parietal F (5, 166) = 8.95, p < .01 regions. Post hoc analysis revealed that these effects were driven primarily by significant reduction of the P300 aspect at 24 hr between baseline and placebo, while at 48 hr, both placebo and Omega were significantly reduced compared to baseline. These data are shown in Figure 8a.

Further analysis examined Q1 and 4 (first five min and last 5 min of the 20 min task) to determine if performance changes that occurred across treatment by quartile were also present in the physiological signal. Repeated measure ANOVA found no significant interaction of time X treatment effect or treatment main effect in Q1 (data not shown), however, a main effect of time occurred in all regions. In Q4, repeated measures ANOVA found treatment X time effects for frontal, F (2,78) = 3.52, p < .05; central, F (2,78) = 3.55, p < .05; but not parietal regions , F (2,78) = 1.7, p = .1885; see Figure 8b).

IR. The average difference of the P300 wave forms for targets versus non-targets were calculated and examined to determine the effect of Omega on a memory task. Repeated measures ANOVAs revealed significant treatment X time effects for the frontal, F (2,78) = 5.31, p < .01; central, F (2,78) = 8.59, p < .001 and parietal regions F (2,78) = 14.80, p < .001 (see figure 9). There were also main effects of time and treatment for each region (ps < 0.05). Post hoc analysis revealed that this was primarily due to the increase in the amplitude of difference wave form in the Omega treatment group for all regions, but only at 24 hr.

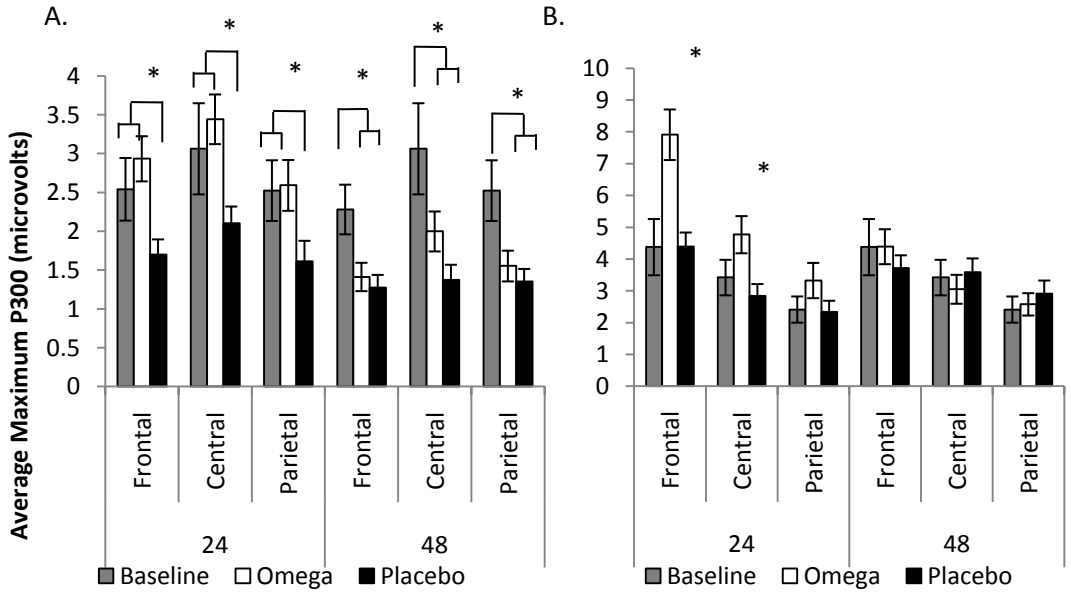

Figure 8. P300 feature maximum amplitude for correct targets for the 3CVT for the entire session (A), quartile 1 (B), and quartile 4 (C). As with performance, ERP components that are significantly altered by Omega appear to develop after the first 5 min of the task.

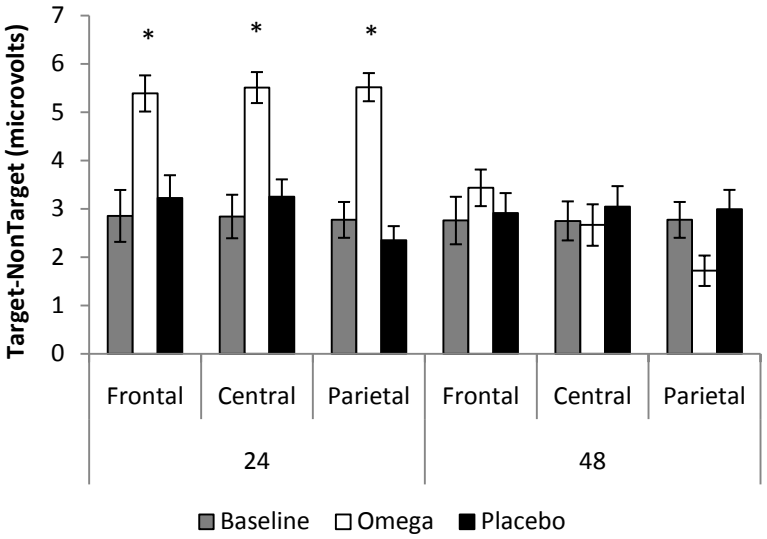

Figure 9. P-300 difference waves from correct targets minus non-targets for the IR task.

3.1.4. Biomarkers/Cytokines. A total of 32 biomarkers were analyzed, however several were consistently below the minimal threshold of the assay, leaving a total of 20 analytes. Separate ANOVAs conducted on individual analytes (with the appropriate Bonferoni adjustments) found that only two analytes were significantly different across conditions (baseline, Placebo, Omega): IL-1 α, IL-1 β, and IL-6. The ANOVA for IL-6 reveled a main effect of treatment, $F(2,81) = 4.47$, $p < .05$, that post hoc analysis further revealed occurred by the Omega treatment maintaining baseline levels of IL-6. The ANOVA for IL-1β revealed a main effect for treatment as well, $F(2,81) = 3.81$, $p < .05$, that post hoc analysis further found was due to a suppression of IL-1β in the Omega treatment group compared to both baseline and placebo. These data are shown in Figure 810, with asterisks (*) denoting significant effects.

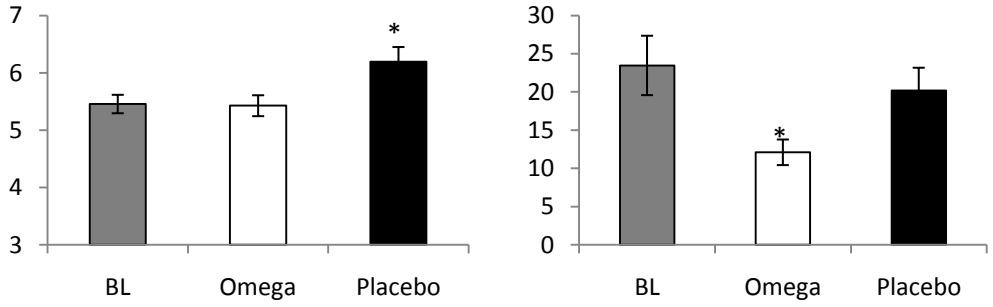

Figure 10. Biomarkers for under acute sleep deprivation of 48 hours. Omega treatment maintained baseline levels of IL-6 (A), and suppressed below baseline and placebo for IL-1 β (B) and below placebo for IL-1 α (C).

3.1.5. Relationships. A stepwise multiple regression was conducted to determine what physiological and self report factors explained performance decrement over the sleep deprivation period, regardless of condition. The Summary of the variables selected are shown in Table 3. Figure 11 presents the variability explained by variable selected in the analysis, with the type of metric color coded by biomarker – red, demographic/self report- orange, P300/ERPS- blue. While biomarkers were rarely significantly different across treatment groups, they explained 17-39% of the variance in performance decrement over time. Self report and demographic metrics explained 32-40%, with the strongest predictor across all performance categories being Vigor (up to 23%).

Table 3

Type	Measure	3CVT RT	3CVT PC	SIR RT	SIR PC
Biomarker	BDNF				X
	Etxn1	X			
	FVII			X	X
	IL18				X
	IL1a	X	X		
	IL1aratio		X		
	IL1b			X	X
	IL1bratio	X			
	IL23	X	X		X
	IL4	X	X		
	IL6				X
	IL7	X	X		
	IL8	X		X	X
	MIP1b		X		
	MMP3				X
	SCF			X	
Demographic	BMI	X	X		
	Age		X		
	Treatment		X		X
Self Report	BDI (depression)	X		X	X
	MOS_Vitality	X	X		
	MOS_Mental Health		X		
	MOS_Pain	X	X		X
	MOS_Physical Role	X			X
	MOS_Social Functioning			X	
	POMS_Anger	X	X		
	POMS_Anxiety		X		
	POMS_Confusion			X	
	POMS_Depression			X	
	POMS_Vigor	X		X	X
	Sleep Quality	X		X	X
	Trait Anxiety			X	
P300	CVT_48Central	X	X		
	CVTQ1_48Parietal	X			
	CVTQ4_24Parietal	X	X		
	SIR24_Frontal			X	
	SIR24_Parietal			X	X
	SIR48_Central				
	SIR48_Parietal			X	

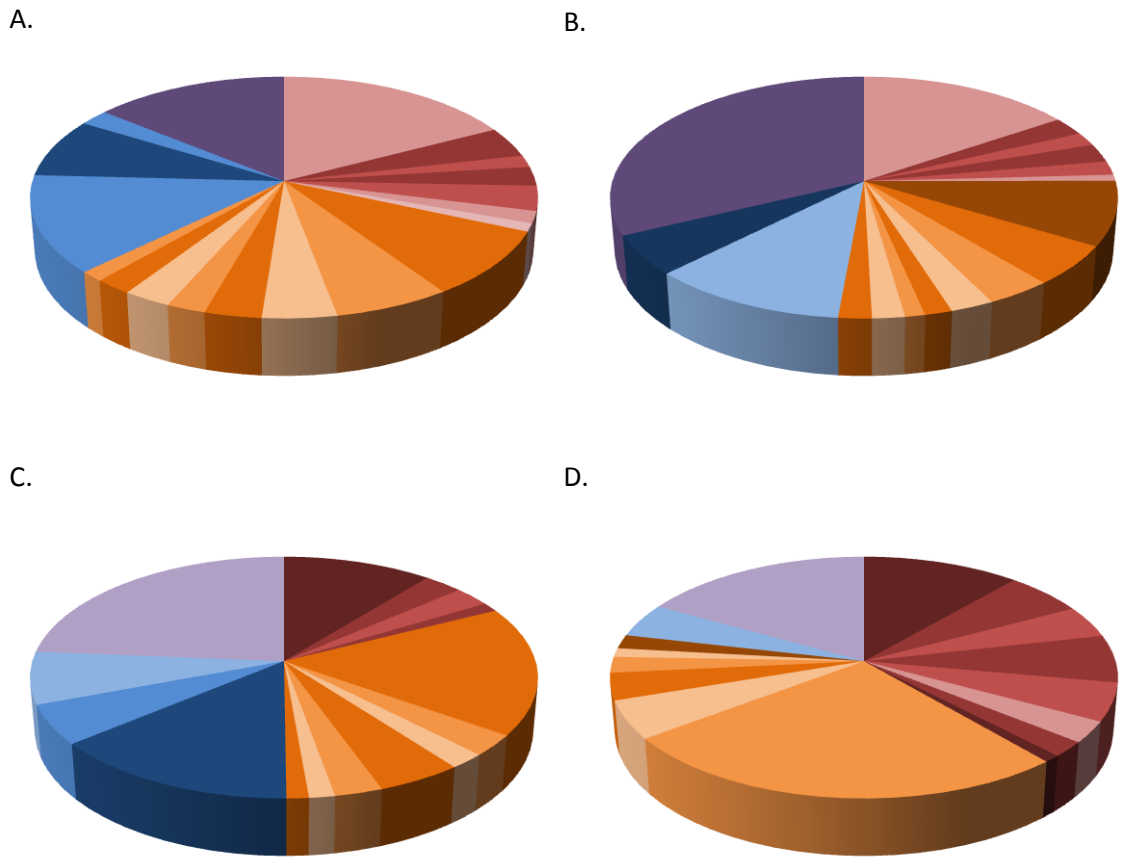

Figure 11. Stepwise regression results for 3CVT speed (A), accuracy (B), and IR Speed (C) and Accuracy (D). Red shades indicates cytokines/biomarkers, Orange shades indicate self report/demographic, blue indicates P300 metrics, and purple is other, unexplained variance

3.2. Chronic

3.2.1. Omega-3 Blood Lipid level. Omega-3 blood lipid profiles were assayed for a subset of participants to confirm the efficacy of the treatment. Mean lipid profile of Omega-3 in the Omega groups was 4.2% +/.6 compared to the placebo group 2.1% +/- .8.

3.2.1. Sleep (actigraphy/self report). Complete records of average sleep were determined by combing both the actigraphy and sleep log data, as well as the self report metric on the PSQI, for baseline. Accumulated sleep deprivation was calculated by taking the baseline

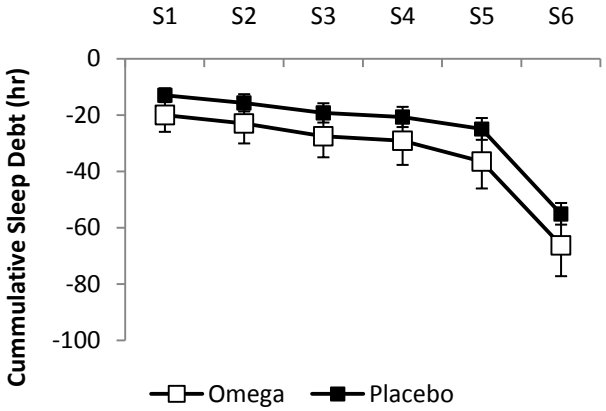

Figure 12. Cumulative sleep loss over time. Marines lost up to 61 hr during the 12 week training

self report as the norm, 6.6 hr. Thus these Marines were already acquiring less than the recommended 7.5 hr minimum, and thus may have begun the study sleep deprived. Repeated ANOVA found no interaction of treatment and time, nor any main effect of treatment on amount of sleep obtained. However, a main effect of time was found for the amount of sleep debt accumulated. At baseline all subjects were acquiring 5.5-7.5 hr per night, with a mean of 6.6 hr. These data are shown in Figure 12. From here forward, graphs are labeled with the average amount of sleep loss based on this analysis.

3.2.3. Self Report Mood/Well Being. Self report metrics from the POMS, BDI, CESD, STAI, MOS-36, and PSQI were analyzed. Overall, there were significantly more differences across groups that found in the acute study. First, while assignment was done randomly, there were differences across the groups at baseline for perceptions of STAI- Anxiety, MOS-Physical Role capability, MOS- Bodily Pain, and POMS- Tension. In addition, all participants scored higher than normal on STAI Anxiety, PSQI (i.e. sleep quality was below average), more than normal perceptions of bodily pain, and lower than normal overall mental health, as well as greater than normal Tension, Anger, and Fatigue. However, even with these baseline differences, the Omega treatment significantly elevated perceptions of vigor, reduced fatigue, and reduced perceptions of overall physical health and bodily pain compared to placebo, regardless of time. In addition, over the course of the training course, the Placebo treatment group had greater perceptions of depressive symptoms, anxiety, bodily pain, tension and anger. These data are summarized in Table 4. Figure 13 presents the main effect of treatment for Fatigue and Vigor for ease of comparison with the acute data.

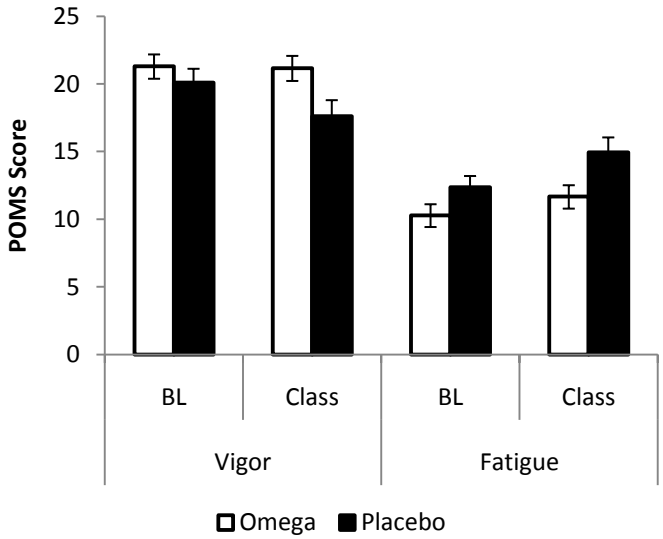

Figure 13. POMS main effects of Vigor and Fatigue. Vigor is consistent with the acute study levels, however fatigue is nearly double, at baseline and subsequent time points.

Table 4.

Measure	Scale	Norm	Omega	Placebo	P(Tx)	P (Ixn)
CESD	Depression	0-16	8.8/10.3	10.3/19.2	NS	.005
STAI	Anxiety	36 +/- 10	65.2/58.7	75/76.7	NS	.020
PSQI	Sleep Quality	0-5			NS	NS
MOS	Physical	>84.2	91.1/96.4	91.4/81.2	.030	.006
	Role- Physical	>80.9	91.0/96.5	81.6/72.6	NS	.030
	Bodily Pain	>75.2	67.8/64	57.6/57	.020	.001
	Vitality	>60.9			NS	NS
	Social Functioning	>83.3			NS	NS
	Role- Emotional	>81.3			NS	NS
	Mental Health	>74.7			NS	NS
POMS	Tension/Anxiety	<8	9.4/4	12.2/13	NS	.001
	Depression	<8	6.38/5.2	10.8/15	NS	.001
	Anger	<8	10.7/10	13.4/20.3	NS	.019
	Vigor	>19	21.3/22.7	20.1/17.3	.004	NS
	Fatigue	<8	10.3/9.6	12.4/17.2	.020	NS
	Confusion	<6.5			NS	NS

3.1.2. Performance. Lapses (failure to respond for 3 s or longer) during the passive auditory (EC) and visual (EO) vigilance tasks were examined. Repeated measures two-way ANOVA (treatment X time) found no significant differences ($p > .05$) over time, across treatment, or any interaction of time and treatment, similar to the failure of this metric to distinguish treatment effects in the acute study.

Accuracy and speed were analyzed for both the 3CVT and the IR tasks using Repeated ANOVA across the fully rested baselines and the eight sleep deprived time points. The repeated ANOVA (2 X 9) revealed that Omega-3 supplementation led to significantly better accuracy on both parameters for 3CVT, $Fs(6,108) = 3.27$, $p < .01$, but not for the IR task. These data are presented in Figure 14.

Based on this, the analysis to determine at what point performance began to decrement was performed only for the 3CVT. For both speed and accuracy, this occurred in session 5 (week 10 of the 12 week class), at an average of 31 hr of sleep deficit, $Fs(1,22) \geq 21.23$, $ps \leq .0001$.

Additional analysis was done across the 4 quartiles of the 3CVT in 4 separate repeated two way ANOVAS (treatment X time), 1 per quartile. A main effect of treatment, $Fs(1,23) \geq 14.62$, $ps \leq .01$ occurred beginning in quartile 2 for both speed and accuracy. However the interaction of treatment X time only occurred in the final quartile, $Fs(6,108) \geq 3.77$, $ps \leq .01$. In contrast, Speed became different in quartile 2, but over the course of the class, this difference dissipated as sleep loss increased. These data are shown in Figure 6.

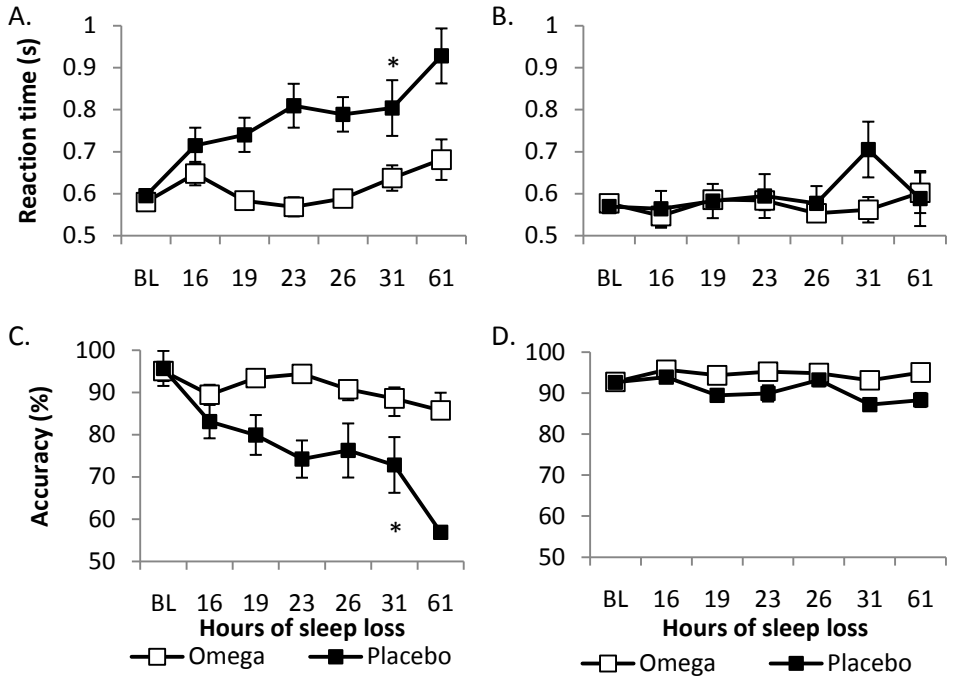

Figure 14. Performance metrics for chronic study 3CVT (A, B) and IR (C, D) including Speed (A, C) and Accuracy (B, D). Asterisks (*) indicate the point at which performance became decremented below that of baseline.

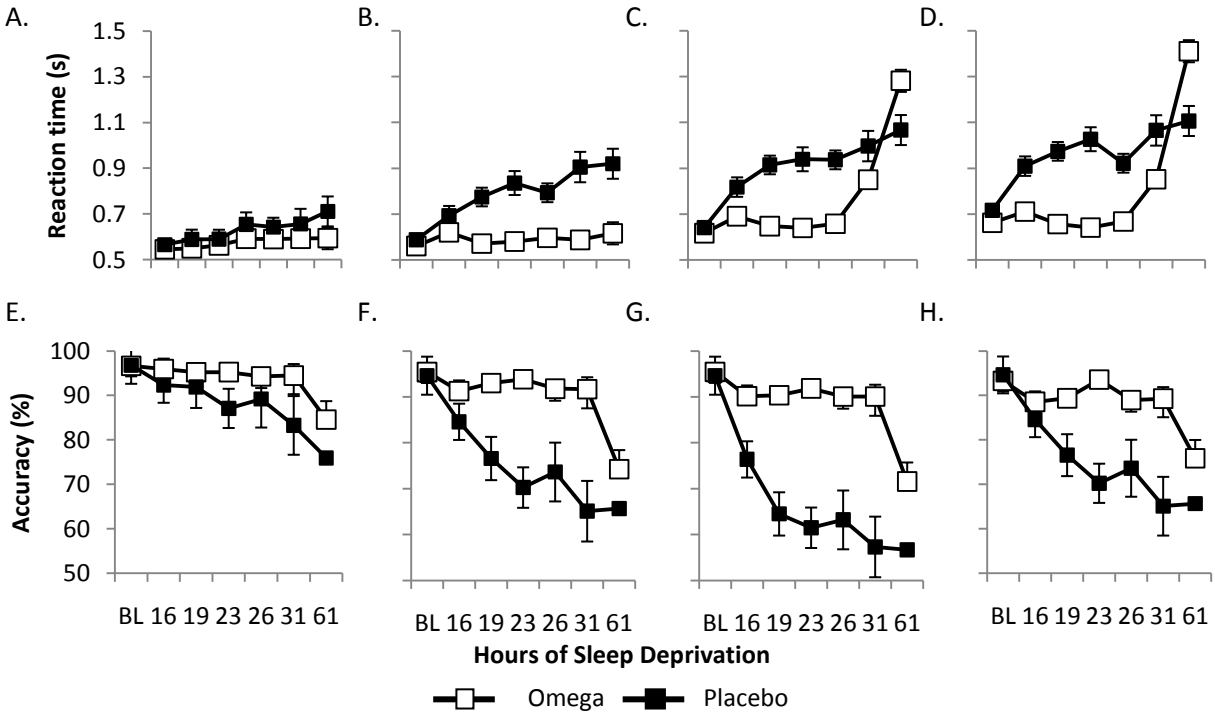

Figure 15. 3CVT performance metrics by quartile for the chronic study (20 min task examined in 5 minute sections). A-D represent speed, E-H accuracy. Effects began in quartile 2.

3.1.3. EEG ERPs.

3CVT. For the 3CVT data, the ERP for the Correct Targets were calculated and the average amplitudes of the P300 feature (between 175-450 ms) were compared across treatments at each time point, for frontal, central, and parietal sites (Figure 16). Repeated ANOVA found no significant effects over time x treatment. However, main effects of treatment were revealed for frontal, F (1,19) = 5.99, p < .02; central, F (1,19) = 11.32, p < .01; and parietal regions , F (1,19) = 6.16, p = .02.

Further analysis examined Q1 and 4 (first five min and last 5 min of the 20 min task) to determine if performance changes that occurred across treatment by quartile were also present in the physiological signal in the chronic study as it did in the acute study. Repeated ANOVA found no significant effects over time x treatment in quartile 1. For quartile 4, main effects of treatment were revealed for frontal, F (1,19) = 5.75, p < .02; central, F (1,19) = 7.55, p < .01; and parietal regions , F (1,19) = 21.94, p = .001 (as with the overall session, so time X treatment effects occurred for either quartile). These data are shown in Figure 16.

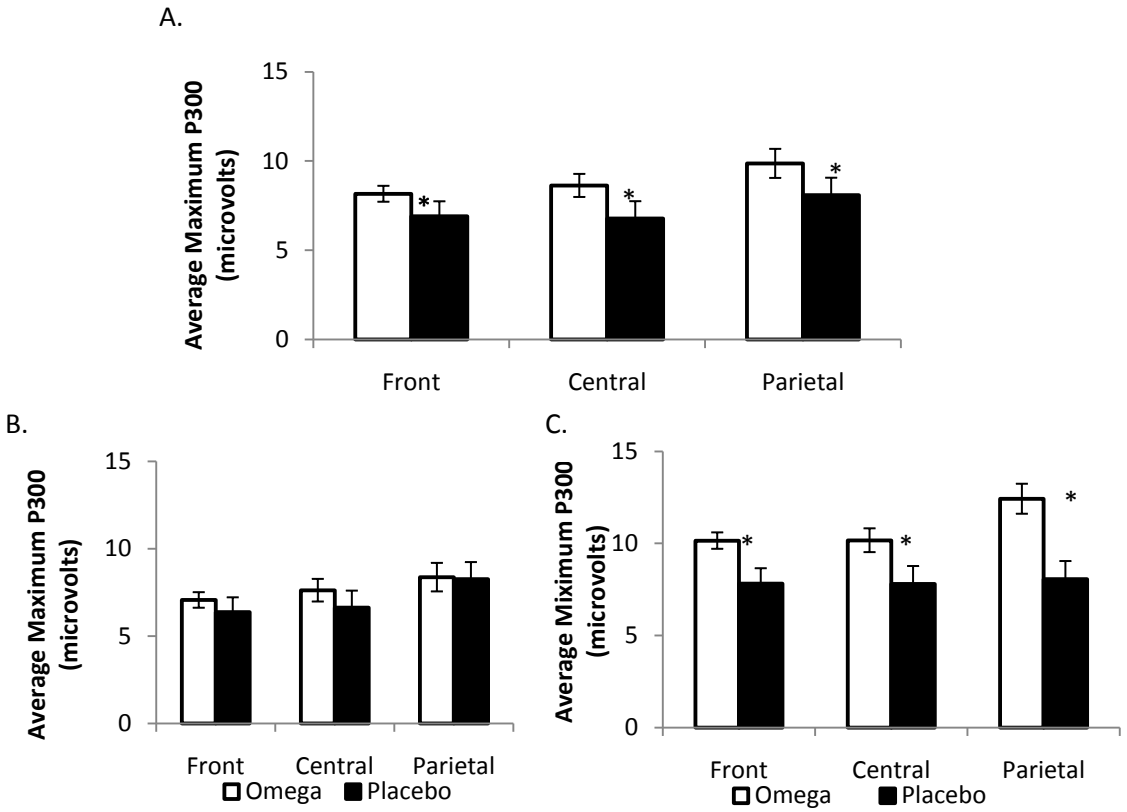

Figure Figure 16. P300 component for 3CVT correct targets for the chronic study. P300 maximal amplitude occurred over the 20 min session (A), and similar to the acute data the P300 component does not appear in quartile 1 (B), but is clear by quartile 4(C).

IR. The average difference of the P300 wave forms for targets versus non-targets were calculated and examined to determine the effect of Omega on a memory task. However, like performance on the IR task in the chronic participants, no significant effects were found for the P3-00 difference wave.

3.1.4. Cytokines. A total of 32 biomarkers were analyzed in blood, and in addition, 7 were analyzed in saliva as well, however several were consistently below the minimal threshold of the assay, leaving a total of 23 analytes. Because of the between subject design for this study, a pre and post treatment sample were acquired. Thus the change from baseline was analyzed. Omega treatment significantly suppressed expression over time for many of the pro-inflammatory analytes (blood IL-18, IL-8, MCP1; salivary IFNγ, IL-1β, IL-2, IL-4, and TNFα), reduced expression to below baseline for blood MIP1b, IL-1α, and salivary IL-6(marginal). Table 5 summarizes these findings.

Table 5

Type	Inflammatory type	Measure	Omega	Placebo	P
Blood	+	IL-18	95.9	175.3	NS
	+	IL-8	17.7	26.5	.0009
	+	MCP1	105.6	147.3	.03
	+	MIP1b	-36.42	12	.0001
	+	MMP2	.97	1.78	NS
	-	BDNF	1.3	1.1	NS
	+	Eotaxin	-59.85	-47.54	NS
	+	ICAM	.35	6.14	NS
	+	IL1α	-.04	-.01	.0002
	+	IL-1β	-13.0	1.08	NS
	+	IL-15	.02	-.05	NS
	+	IL-1ra	2385	2475	NS
	+	IL-23	.09	.15	NS
	+	MMP3	-2.11	-3	NS
	-	SCF	10	6.8	NS
	-	VEGF	342	335	NS
Saliva	+	IFNγ	.08	.85	.04
	+	IL-1β	3.6	6.7	.05
	+	IL-2	1.6	6.0	.02
	+	IL-4	.006	.45	.01
	+	IL-6	-.45	.82	NS(.08)
	+	TNFα	1.24	1.25	NS(.09)

3.1.5. Relationships. As with the acute study, we examine the relationship between physiological and self report measures in explaining the change in performance over time for 3CVT and IR accuracy and speed. Metrics that were not significant in either study across treatment groups, such as heart rate and heart rate variability were found to explain variance in performance with these analysis. It should simply be noted that there were no effects for these metric in other analysis outside of the stepwise regression. As with the acute data, biomarker cytokine activity was significant, as were self report metrics. In contrast, very few metrics were found to be required to explain the variance in these participants for each of these performance metrics (as few as 3 or 4 for each). Figure 17 describes the results of this analysis.

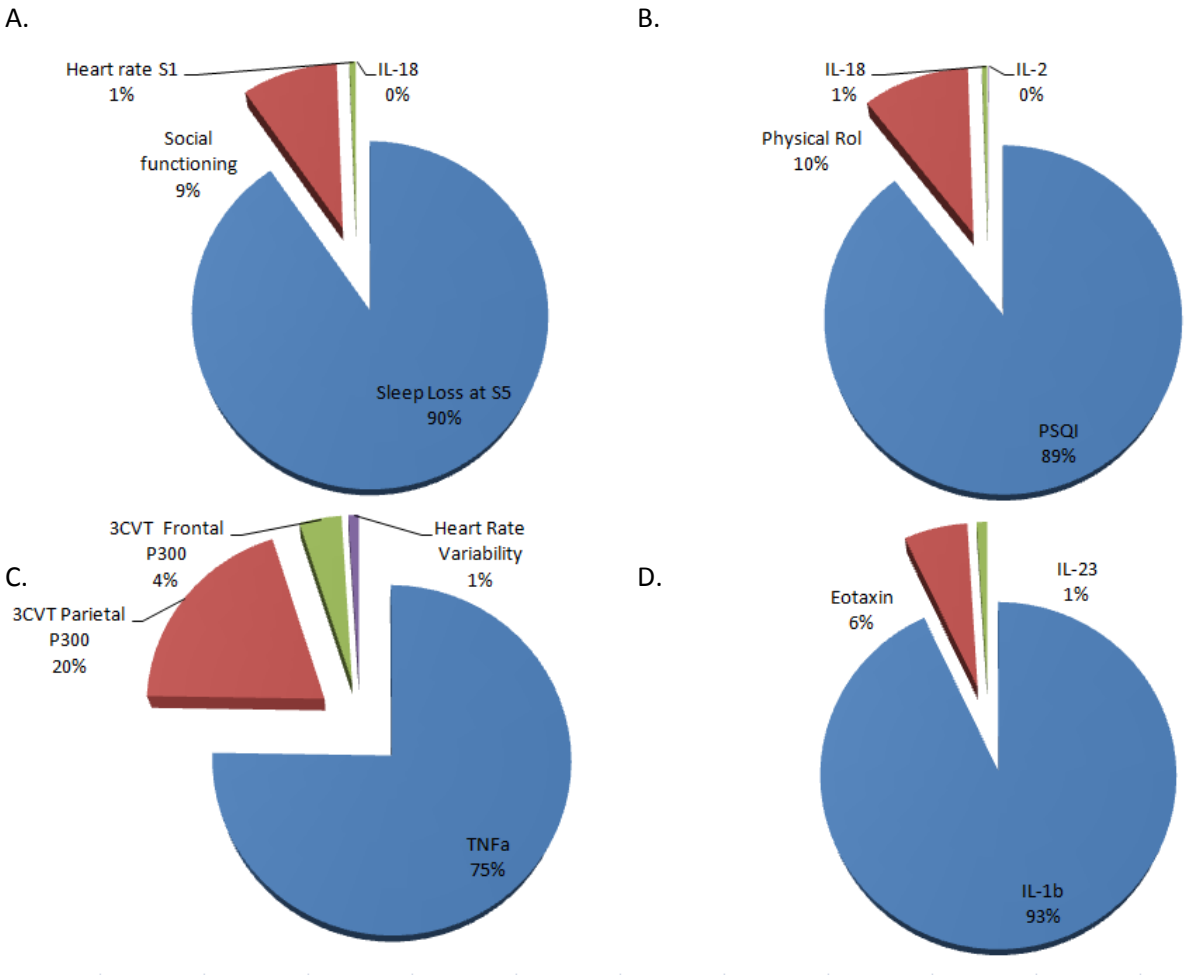

Figure 17. Stepwise analysis reveals that , unlike in the acute study, the decrement in performance over the chronic study was explained by fewer variables (no more than 4), and one variable primarily explains the change in performance for each metric: 3CVT speed (A), 3CVT accuracy (C), IR speed (B) and accuracy (D). Inflammatory cytokines primarily explain accuracy on both tasks.

3.3. Acute vs. Chronic

Table 6 presents the best fit lines to calculate how acute and chronic sleep loss relate, based on the performance metrics for 3CVT and IR. Acute sleep deprivation often leads to a sharper decrement in performance, as would be expected. Figure 18 presents a composite standard curve based on these equations that can be used to determine how acute deprivation relates to chronic debt accumulation. Speed and accuracy appear to have somewhat different relationships compared across acute and chronic sleep deprivation, and are therefore given separate presentation. The average best fit line is shown to provide an easy calculation for any future data to translate across acute and chronic sleep deprivation data.

Table 6

Metric	Acute	Chronic
3CVT Speed	Y=0.0078x+0.435	Y=0.0014x+0.6286
3CVT Accuracy	Y=-0.1074x+71.309	Y=-0.7845x+89.51
IR Speed	Y=0 .0101x+0.3763	Y=0.0005x+0.5902
IR Accuracy	Y=-0.1901x+93.562	Y=-0.9324x+114.61

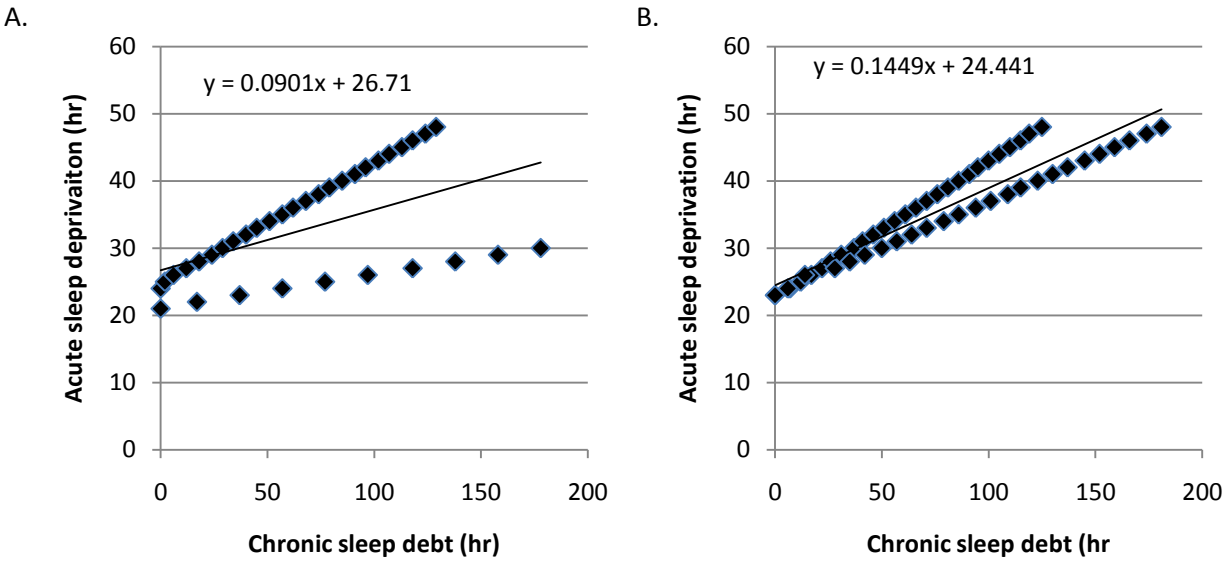

Figure 18. Acute vs. chronic translation/standard curves for speed (A) and accuracy (B).

IV. Conclusions

The data gathered in the course of this work indicate that Omega-3 supplementation can preserve baseline performance on neurocognitive tasks for up to 39 hr of acute sleep deprivation, and 31 hr of chronically accumulated sleep debt. The chronic data must be interpreted with the understanding that the participants reported acquiring less than the minimum recommended sleep at baseline (6.6 hr), and that sleep dent was calculated based on the self reported norm. Thus the performance decrements may have been more prominent earlier in both conditions if these participants had begun the study fully rested.

The performance decrement patterns were supported by commensurate alterations in the p300 component of the ERPs for each task. In the 3CVT, in both studies, performance began to decrement most after the first 5 min of the 20 min task. The P300 amplitudes also did not show a difference until after the first 5 min. This is of note when we examine the ANAM data. The ANAM tasks are highly abbreviated, lasting only 5 min at the longest. Thus such short tasks may not be sensitive to the effects of these types of interventions. In contrast, we also found that shorter IR task (6 min) was able to distinguish across treatment groups in the acute study. This may be due to the more complex nature of the task that pushes short term memory to recognize 20 memorized targets.

In addition to the physiological metrics, the biomarker/cytokine metrics also demonstrated a reduction or suppression of inflammatory response in both studies in the Omega group. These effects were more evident in the chronic study, and may be due to the fact that all participants in the chronic study were supplemented a total of 12 weeks, through the course, while the acute subjects were supplemented a maximum of 6 weeks. Regardless, these data support the hypothesis that the inflammatory system is playing a mediating role in the effects of sleep loss, whether chronic or acute, and that modulating the inflammatory response through Omega-3 supplementation is effective at both reducing overall inflammation and preserving performance.

The blood lipid profiles indicate that the supplementation was effective at increasing physiologically available Omega-3. However, even after up to 12 weeks of supplementation, the participants in both studies remained well below the recommended level of 7%, with the placebo group at well below the minimal level required for heart health, 3% (93). Given the time it takes to improve blood lipid profiles through supplementation alone, most researchers recommend not only increasing Omega-3 foods (cold water fatty fish, tree nuts, flax) but also reducing the amount of high Omega-6 (pro-inflammatory) foods (fried foods, grain fed meats, peanuts). These data support that recommendation.

Inflammatory markers and self report/demographics explained the majority of the variance in performance decrement, with the cleanest and clearest relationships revealed in the chronic study. Thus, in addition to Omega-3 supplementation to reduce inflammation, interventions that improve perceptions of self efficacy (i.e. vigor, role functioning) may also improve performance preservation when sleep deprived.

Based on these data, Omega-3 supplementation for the military is supported to improve cognitive functions and mood perception when soldiers are likely to undergo periods of acute or chronic sleep loss. However, improving the dietary Omega-3:Omega6 intake would also be advantageous.

The majority of research on sleep loss is conducted using the acute sleep deprivation model, while the majority of daily activities, and certainly the majority of military tasks occur under conditions of chronic sleep debt, accumulated sleep loss over time. We were able to take advantage of the parallel design of these studies to develop standardized curves to compare acute studies, and how they relate to chronic sleep deprivation. It should be noted that the participants used to develop these curves were likely already somewhat sleep deprived, and thus more likely to correlate to the common soldier and sailor.

This study allowed us to meet our three objectives: 1) assess the effectiveness of Omega-3 fatty acids in mitigating the effects of acute and chronic sleep deprivation, 2) assess inflammatory activity throughout both acute sleep deprivation in order to begin to determine if the effects of sleep loss are primarily mediated through inflammation, 3) compare how acute and chronic sleep loss compare on identical metrics to develop “standardized curves” for use by military leaders. However, we do not know how many hours of recovery sleep, or how many days of “normal” sleep with or without Omega-3 supplementation are required to return a sleep deprived soldier or sailor back to baseline performance levels. Nor do we have any metrics on the efficacy of this intervention on preserving performance on military relevant tasks. Future work should address these issues.

V. References

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